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Inventors (please provide full names): GARY P. Nolan and

Donald PAYAN

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Scaffold => Read as DNA or RNA?

Binding sites => sites for DNA/RNA Related enzyme
ie. {DNA Polymerase
{RNA Polymerase
transcription factors.

TARGETING SEQUENCE=>ie NLS, Golgi Sequence, Mitochondrial etc.

claims 1-8, 28 & 32, 32 39 & 39 are elected

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I think this Reads on a virus being actively Replicated in a cell. The virus would have to be Virus (Adeno virus for example) as required by Claim 28.

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PTO-1590 (2-99)

L4 ANSWER 1 OF 3 CAPLUS COPYRIGHT 2002 ACS
AN 1996:534930 CAPLUS
DN 125:189269
TI Evidence for Two Catalytically Independent Clusters of Active Sites in a Functional Modular **Polyketide Synthase**
AU Kao, Camilla M.; Pieper, Rembert; Cane, David E.; Khosla, Chaitan
CS Department of Chemical Engineering, Stanford University, Stanford, CA, 94305-5025, USA
SO Biochemistry (**1996**), 35(38), 12363-12368
CODEN: BICHAW; ISSN: 0006-2960
DT Journal
LA English
AB Modular **polyketide synthases** (PKSs), such as the 6-deoxyerythronolide B **synthase** (DEBS), catalyze the biosynthesis of structurally complex and medicinally important natural products. These large multifunctional enzymes are organized into modules, where each module contains active sites homologous to those of higher eukaryotic fatty acid synthases (FASs). Like FASs, modular PKSs are known to be dimers. Here we provide functional evidence for the existence of two catalytically independent clusters of active sites within a modular PKS. In three bimodular derivs. of DEBS, the ketosynthase domain of module 1 (KS-1) or module 2 (KS-2) or the acyl carrier protein domain of module 2 (ACP-2) was inactivated via site-directed mutagenesis. As expected, the purified proteins were unable to catalyze polyketide synthesis (although the KS-1 mutant could convert a diketide thioester into the predicted triketide lactone). Remarkably however, the KS-1/KS-2 and the KS-2/ACP-2 mutant pairs could efficiently complement each other and catalyze polyketide formation. In contrast, the KS-1 and ACP-2 mutants did not complement each other. On the basis of these and other results, a model is proposed in which the individual modules of a PKS dimer form head-to-tail homodimers, thereby generating two equiv. and independent clusters of active sites for polyketide biosynthesis. Specifically, each subunit contributes half of the KS and ACP domains in each cluster. A similar complementation approach should also be useful in dissecting the organization of the remaining types of active sites within this family of multienzyme assemblies. Finally, blocked systems, such as the KS-1 mutant described here, present a new strategy for the noncompetitive conversion of unnatural substrates into polyketides by modular PKSs.

L4 ANSWER 2 OF 3 CAPLUS COPYRIGHT 2002 ACS
AN 1995:732738 CAPLUS
DN 123:137313
TI Dissociation of dimeric 6-hydroxymellein **synthase**, a **polyketide** biosynthetic enzyme in carrot cell extracts, with loss of keto-reducing activity
AU Kurosaki, Fumiya
CS Lab. Cell Biol., Toyama Med. Pharmaceutical Univ., Toyama, 930-01, Japan
SO Arch. Biochem. Biophys. (**1995**), 321(1), 239-44
CODEN: ABBIA4; ISSN: 0003-9861
DT Journal
LA English
AB 6-Hydroxymellein **synthase**, an inducible **polyketide** biosynthetic enzyme in carrot cell exts., is composed of two identical

subunits, and the homodimer is dissocd. to monomeric peptides under high-ionic-strength conditions with loss of the **synthase** activity. Appreciable radioactivities were assocd. with the synthase proteins when the monomer enzyme was incubated with the radiolabeled substrates, acetyl-CoA and malonyl-CoA. Therefore, it appeared that the synthase does not lose the ability of binding the substrate even after the dissocn. to monomers. The monomeric synthase liberated triacetic acid lactone as the derailment product instead of 6-hydroxymellein from the enzyme-attached triketomethylene chain which is the immediate precursor of an NADPH-dependent keto-reducing reaction involved in 6-hydroxymellein biosynthesis. These observations strongly suggest that the monomeric synthase retains the ability of ketomethylene chain elongation by the condensation of acyl-CoAs, but is lacking in an NADPH-dependent keto-reducing activity toward the triketide intermediate. Results obtained in the present expts. imply that the catalytic domain of acyl-CoA condensation is able to assoc. with that of keto redn., possibly belonging to another subunit, only in the homodimeric structure to organize the multicatalytic reaction center.

L4 ANSWER 3 OF 3 EMBASE COPYRIGHT 2002 ELSEVIER SCI. B.V.
AN 96049686 EMBASE
DN 1996049686
TI Evidence for a double-helical structure for modular **polyketide synthases**.
AU Staunton J.; Caffrey P.; Aparicio J.F.; Roberts G.A.; Bethell S.S.; Leadlay P.F.
CS Department of Biochemistry, University of Cambridge, Tennis Court Road,Cambridge CB2 1QW, United Kingdom
SO Nature Structural Biology, (1996) 3/2 (188-192).
ISSN: 1072-8368 CODEN: NSBIEW
CY United States
DT Journal; Article
FS 029 Clinical Biochemistry
LA English
SL English
AB Modular **polyketide synthases** are multienzymes responsible for the biosynthesis of a large number of clinically important natural products. They contain multiple sets, or modules, of enzymatic activities, distributed between a few giant multienzymes and there is one module for every successive cycle of **polyketide** chain extension. We show here that each multienzyme in a typical modular **polyketide synthase** forms a (possibly helical) parallel dimer, and that each pair of identical modules interacts closely across the dimer interface. Such an arrangement would allow identical modules to share active sites for chain extension, and thus to function independently of flanking modules, which would have important implications both for mechanisms of evolution of **polyketide synthases** and for their future genetic engineering.

ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS
Vol. 321, No. 1, August 1, pp. 239–244, 1995

Dissociation of Dimeric 6-Hydroxymellein Synthase, a Polyketide Biosynthetic Enzyme in Carrot Cell Extracts, with Loss of Keto-Reducing Activity

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6-Hydroxymellein synthase, an inducible polyketide biosynthetic enzyme in carrot cell extracts, is composed of two identical subunits, and the homodimer is dissociated to monomeric peptides under high-ionic-strength conditions with loss of the synthase activity. Appreciable radioactivities were associated with the synthase proteins when the monomer enzyme was incubated with the radiolabeled substrates, acetyl-coenzyme A (CoA) and malonyl-CoA. Therefore, it appeared that the synthase does not lose the ability of binding the substrate even after the dissociation to monomers. The monomeric synthase liberated triacetic acid lactone as the derailment product instead of 6-hydroxymellein from the enzyme-attached triketomethylene chain which is the immediate precursor of an NADPH-dependent keto-reducing reaction involved in 6-hydroxymellein biosynthesis. These observations strongly suggest that the monomeric synthase retains the ability of ketomethylene chain elongation by the condensation of acyl-CoAs, but is lacking in an NADPH-dependent keto-reducing activity toward the triketide intermediate. Results obtained in the present experiments imply that the catalytic domain of acyl-CoA condensation is able to associate with that of keto reduction, possibly belonging to another subunit, only in the homodimeric structure to organize the multicatalytic reaction center. © 1995 Academic Press, Inc.

Key Words: **polyketide biosynthesis; keto-reducing activity; multifunctional enzyme; homodimer; 6-hydroxymellein synthase; carrot (*Daucus carota* L.).**

It is well known that many higher plants produce a variety of secondary metabolites by the head-to-tail condensation of acetyl-CoA[2] and malonyl-CoA; a polyketomethylene chain is postulated as a hypothetical intermediate involved in the synthetic reactions (1). Biosynthetic enzymes that produce this class of compounds, polyketides, have been assumed to share many common properties with fatty acid synthases, because most of the partial reactions involved in the two processes are similar. However, unlike fatty acid synthases, the biochemical properties of the polyketide synthetic enzymes have not been well defined sinc these enzymes are usually very unstable (1, 2). It has been shown that higher plant and bacterial fatty acid synthases can be readily separated into the individual catalytic units by conventional procedures (type II) (3), while animal and yeast fatty acid synthases exist as multifunctional enzymes (type I) (3, 4). The latter are further divided into two subclasses, IA (consisting of two identical subunits) and IB (in higher states of aggregation) (4). By contrast, there have been very few attempts to elucidate the structural organization of polyketide biosynthetic enzymes in plants (1, 2).

It was shown (5) that carrot cells as an induced resistance mechanism accumulate 6-methoxymellein (Fig. 1, 2), a pentaketide compound with antifungal activity, upon invasion by pathogenic microorganisms. We have demonstrated (6) that 6-hydroxymellein (1) is the direct precursor of 6-methoxymellein and that the former compound is synthesized by the condensation of 1 acetyl-CoA and 4 malonyl-CoA. It was also reported (6) that an NADPH-dependent keto reduction of a carbonyl group takes place at the triketide stage to form the reduced ketomethylene chain as an important intermediate (Fig. 1). It has been shown that biosynthesis of 6-methoxymellein is catalyzed by two inducible enzymes, 6-hydroxymellein synthase (6) and 6-hydroxymellein-*O*-methyltransferase (7). It has been shown (8) that 6-hydroxymellein synthase is a multifunctional enzyme, which resembles type I fatty acid synthases,

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[2] Abbreviations used: CoA, coenzyme A; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SH, sulfhydryl.

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and catalyzes the series of reactions involved in the biosynthesis of the compound. More recently, I have reported (9) that 6-hydroxymellein synthase is organized as a homodimer and the two subunits are quantitatively dissociated to monomers with loss of the synthase activity under the high-ionic-strength conditions in a reversible manner. This fact also implies that the functional structures in the synthase subunit are not destroyed by dissociation of the native dimer and suggests the possibility that the monomer merely loses some of the partial activities essential for 6-hydroxymellein biosynthesis. In the present paper, I provide evidence that the NADPH-dependent keto-reducing activity of 6-hydroxymellein synthase toward the triketide intermediate is lost upon dissociation of the native homodimer to monomeric subunits. Possible arrangement of catalytic domains of the synthase in the active dimer structure is also discussed.

MATERIALS AND METHODS

Chemicals. 6-Methoxymellein was isolated from fungus-infected carrot root tissues (5), and 6-hydroxymellein was prepared by demethylating the compound with BBr_3 in anhydrous methylene chloride as reported previously (10). Triacetic acid lactone was synthesized from dehydroacetic acid (Nacalai Tesque) according to the method of Collie (11). 2-Chloroethylphosphonic acid, acetyl-CoA, malonyl-CoA, NADPH, and bovine serum albumin were purchased from Sigma. [2-^{14}C]Acetyl-CoA (sp act, 2.1 GBq/mmol) and [2-^{14}C]malonyl-CoA (sp act, 2.2 GBq/mmol) were from New England Nuclear. All other chemicals were reagent grade.

Induction and purification of 6-hydroxymellein synthase. Carrot roots were purchased from a local market, and induction of 6-hydroxymellein synthase activity in root disks was carried out using 2-chloroethylphosphonic acid as the elicitor as described previously (8, 12). Highly purified 6-hydroxymellein synthase was prepared essentially according to the methods described previously (12); however, the chromatofocusing step was omitted in the present experiments. Purity of the synthase preparation was assessed by SDS–PAGE analysis (8% gel) according to the method of Laemmli (13). A densitometric scan on a dual-wavelength chromatoscanner (Shimadzu, CS-910) was performed after the proteins were stained with Coomassie brilliant blue (12). It was confirmed that the synthase comprised 42–63% of the proteins in the enzyme preparation in repeated experiments.

Binding of acyl-CoAs to monomeric and dimeric 6-hydroxymellein synthase. Monomeric 6-hydroxymellein synthase was prepared by the treatment of the native dimeric enzyme with 2 M NaCl according to a method described elsewhere (9). In brief, highly purified 6-hydroxymellein synthase (approximately 60 μg proteins) was dissolved in 2 ml of 20 mM Na–phosphate buffer (pH 7.5) containing 0.2% (v/v) mercaptoethanol. Aliquots (0.8 ml each) of the enzyme solution were transferred into cellophane tubes and were dialyzed at 4°C for 2 h against the same buffer or the buffer containing 2 M NaCl. A portion of each enzyme preparation was further incubated with 10 mM $HgCl_2$ at 37°C for 10 min. Volume of the enzyme solutions was then readjusted to 1 ml, and they were incubated with radiolabeled acyl-CoAs. The assay mixture consisted of 10 mM Na–phosphate (pH 7.5), 50 μM [^{14}C]acetyl-CoA (1.85 kBq), 5 μM malonyl-CoA, 1 mM NADPH, 5 μg protein of the enzyme preparation (approximately 100 pkat/assay), and 0.1% mercaptoethanol in a total volume of 200 μl. In a parallel experiment, [^{14}C]acetyl-CoA and malonyl-CoA were replaced by 50 μM unlabeled acetyl-CoA and 5 μM [^{14}C]malonyl-CoA (1.85 kBq). For the binding assay of the monomeric enzyme, incubation was carried out in the presence of 2 M NaCl. The reaction was run for 30 min at 37°C unless otherwise noted and was terminated by the addition of 0.5 ml of 2 M trichloroacetic acid. To the solution was then added 100 μg of bovine serum albumin as a carrier, and the proteins were precipitated by centrifugation (1000*g* for 5 min). Resultant pellets were successively washed with 1 ml of the trichloroacetic acid solution and the same volume of acetone. The samples were then denatured by boiling for 2 min in the presence of 5% (v/v) mercaptoethanol and 2% (w/v) SDS and were subjected to SDS–PAGE (8% gels). It has been shown (9) that the dimeric enzyme is completely dissociated to the monomeric subunits under these conditions. After electrophoresis, proteins were stained, and the band corresponding to the 6-hydroxymellein synthase subunit (128 kDa) was identified with standard proteins (myosin, 200 kDa; *Escherichia coli* galactosidase, 116 kDa; rabbit muscle phosphorylase, 97 kDa; bovine serum albumin, 66 kDa; ovalbumin, 43 kDa). Gels slices containing the enzyme protein were excised with a blade and were immersed in 1 ml of Protosol (New England Nuclear). Radioactivity involved in the gels was determined after mixing with 10 ml of a commercial scintillation cocktail (Amersham, ACS II).

HPLC analysis of the derailment product liberated by monomeric 6-hydroxymellein synthase. Derailment product liberated by monomeric 6-hydroxymellein synthase was analyzed primarily by HPLC (Hitachi Model 655 equipped with a data processor 655-60) using a reversed-phase C_{18} column (Gasukuro Kogyo Inc., Japan; Unisil Q, 4.6 × 15 cm) according to the method described previously (6). Dimeric and monomeric 6-hydroxymellein synthase were incubated with unlabeled acyl-CoAs in the presence of NADPH for 1 h as described above, and the reaction products were extracted with 0.5 ml of ethyl acetate by blending. The organic phase was removed and evaporated, and the sample was redissolved in 100 μl of methanol. An aliquot (10 μl) was injected onto the HPLC column, and relatively polar compounds were separated with 100% H_2O as the eluting solvent. The elution of the products was monitored by spectrophotometry at 200 nm for the diketide and at 280 nm for the triketide products (6). For analysis of less-polar compounds (tetra- and pentaketide products), the column was eluted with a linear gradient of methanol (30–70%) in H_2O over 20 min with monitoring at 280 nm (6).

RESULTS

Binding of Acyl-CoAs to Monomeric and Dimeric Forms of 6-Hydroxymellein Synthase

The sequence of the reaction catalyzed by 6-hydroxymellein synthase can be described as (i) binding the substrate acyl-CoAs, (ii) elongation of the ketomethylene chain by the condensation of acyl-CoAs, and (iii) keto reduction at the triketide intermediate stage (6). It is possible that the monomer subunits retain or lose some of these partial activities essential for 6-hydroxymellein biosynthesis. To examine whether the monomeric form of 6-hydroxymellein synthase retains the ability to bind the substrates, the monomeric synthase was incubated with [^{14}C]acetyl-CoA plus unlabeled malonyl-CoA or unlabeled acetyl-CoA plus [^{14}C]-malonyl-CoA. This enzyme subunit was then separated from minor contaminant proteins by SDS–PAGE, and radioactivity associated with the enzyme protein was determined. Appreciable radioactivity was found to comigrate with the monomeric synthase as well as with the native dimer when determined under nondissociat-

TABLE I

Comparison of the Relative Molar Ratio of Acetyl-CoA and Malonyl-CoA Bound to 6-Hydroxymellein Synthase

	Acetyl-CoA	Malonyl-CoA
Dimer	1.00	2.21 ± 0.18
$HgCl_2$-treated dimer	0.09 ± 0.05	0.08 ± 0.08
Monomer	0.55 ± 0.05	0.98 ± 0.08
$HgCl_2$-treated monomer	0.04 ± 0.01	0.07 ± 0.03

Note. Highly purified 6-hydroxymellein synthase was treated with NaCl-containing buffer to dissociate the homodimer to the monomeric subunits. Dimeric and monomeric synthases were then incubated with [^{14}C]acetyl-CoA plus unlabeled malonyl-CoA or unlabeled acetyl-CoA plus [^{14}C]malonyl-CoA. After separation by SDS–PAGE, radioactivities associated with the enzyme proteins were determined. Results were expressed as relative molar ratios of the acyl-CoAs in which acetyl-CoA bound to dimeric 6-hydroxymellein synthase was taken as 1.00. The experiments were repeated three times independently, and the means and SD values are presented.

ing conditions (Table I). By contrast, radioactivity associated with either monomer or dimer forms of the synthase markedly decreased when the preparations were pretreated with $HgCl_2$, a potent inhibitor of this SH enzyme (12). This result suggested that the label for the acyl-CoAs that comigrated with the enzymes was not nonspecifically associated with the protein but probably was attached to the active site. Therefore, it is likely that the monomer subunit of 6-hydroxymellein synthase does not lose the ability to covalently bind substrates.

The binding ability of the enzyme toward its substrates varied appreciably, probably because part of the unstable activity (12) was lost during purification. Therefore, an attempt to estimate the molar ratio of enzyme-bound acyl-CoAs versus total enzyme molecules was unsuccessful. By contrast, the ratio of enzyme-bound acetyl-CoA versus malonyl-CoA showed a fairly constant value throughout the experiments. Therefore, the results were expressed as the molar ratio of the acyl-CoAs bound to the monomeric or dimeric form of the enzyme. In each set of the experiments, acetyl-CoA bound to the dimeric synthase molecule was taken as 1.00, assuming that the dimeric enzyme was quantitatively dissociated to two monomeric subunits in the buffer containing 2 M NaCl (9). As shown in Table I, the relative molar ratio of enzyme-bound acetyl-CoA and malonyl-CoA was similar in the monomeric and dimeric forms (roughly 1:2). Since active 6-hydroxymellein synthase should bind one acetyl-CoA plus zero to four malonyl-CoA in the course of the reaction cycle (Fig. 1), this result implies that the enzyme proteins which bind the triketide intermediate are the most abundant species in either dimeric or monomeric form. It appeared, however, that the dimeric enzyme was able to bind approximately twofold more of each substrate compared with the inactive monomer, suggesting that the binding capacity of the subunit does not change even after its dissociation from the active dimeric form.

Identification of Derailment Product Liberated by Monomeric 6-Hydroxymellein Synthase

If the monomer synthase does not lose the ability to condense substrate acyl-CoAs, it is possible that abnormal product(s) synthesized from the oligoketomethylene chain might be liberated by the dissociated enzyme instead of 6-hydroxymellein. Therefore, the formation of a derailment product liberated by monomeric 6-hydroxymellein synthase was sought. Dimeric and monomeric 6-hydroxymellein synthases were incubated with nonlabeled acyl-CoAs in the presence of NADPH, and the reaction products were analyzed by HPLC (Fig. 2). HPLC separation was first performed with H_2O as the eluting solvent to analyze relatively polar compounds synthesized from di- and triketomethylene chains (6), and only one product peak was observed in the sample produced by monomeric synthase (Fig. 2a). The retention time of the compound was identical to authentic triacetic acid lactone (Fig. 1, 3), and this compound was not found in the reaction products formed by the dimeric enzyme (Fig. 2b). Analysis of the reaction product mixture was also carried out with a linear gradient of 30–70% methanol in H_2O to detect less-polar compound(s) possibly formed from tetra- and pentaketomethylene chains (6); however, no significant products were observed in the mixture generated by the monomer enzyme (Fig. 2c). By contrast, the normal product, 6-hydroxymellein, was observed in the mixture formed by the dimeric synthase under these conditions (Fig. 2d). The reaction product liberated by the monomer enzyme was isolated by HPLC and was further analyzed by ultraviolet spectrometry. The spectra of authentic triacetic acid lactone (6) and the derailment product were found to be identical (data not shown). In TLC analysis, the mobility of these two compounds on silica gel was also identical (R_f 0.45, with chloroform:acetic acid, 5:1, v/v), and no other product was observed, except at the origin. The identity of the derailment product as triacetic acid lactone was further confirmed by cocrystallization. Radiolabeled derailment product, prepared by incubation of the monomer enzyme with acetyl-CoA and [^{14}C]malonyl-CoA, was diluted with 60 mg of authentic triacetic acid lactone and repeatedly recrystallized to a constant specific activity of 49.8 ± 3.5 dpm/mg (mean and SD obtained from four times recrystallization).

Since the 6-hydroxymellein synthase preparation employed in the present experiments was not composed of the homogenous protein, the possibility still remained that the triacetic acid lactone identified might

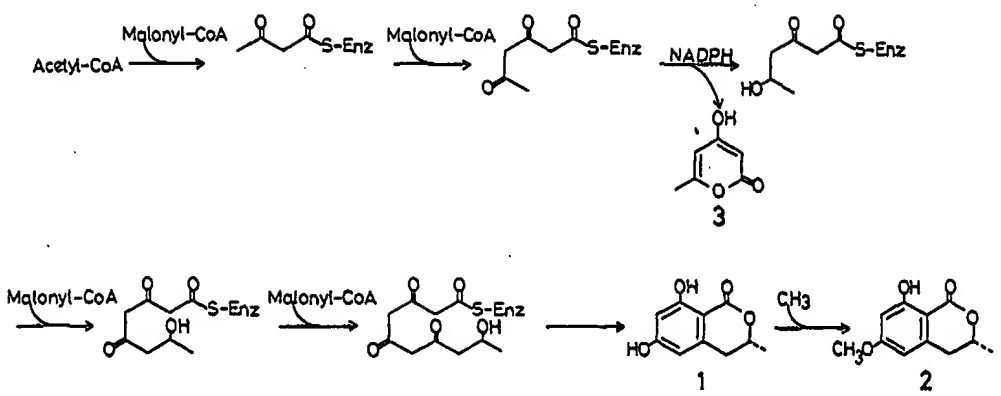

FIG. 1. Biosynthesis of 6-hydroxymellein and 6-methoxymellein. 6-Hydroxymellein synthase catalyzes the condensation of acetyl-CoA and malonyl-CoA. An NADPH-dependent keto reduction takes place at the triketide intermediate stage to form a reduced ketomethylene chain. Further condensation of malonyl-CoA results in the production of 6-hydroxymellein (**1**), and a methyl transfer catalyzed by 6-hydroxymellein-*O*-methyltransferase leads to 6-methoxymellein (**2**). When the keto-reducing reaction does not take place, triacetic acid lactone (**3**) is liberated as the derailment product.

be a product of a contaminating enzyme. To exclude this possibility, dimeric and monomeric 6-hydroxymellein synthase preparations were subjected to SDS–PAGE after being incubated with radiolabeled acyl-CoAs, and the gels were cut into 5-mm slices. Radioactivity of the gels at positions other than the position of 6-hydroxymellein synthase subunits was found to be less than 5% of those associated with the synthase protein, suggesting that there was no contaminating enzyme which utilized acyl-CoA as substrate. In another experiment, utilization of malonyl-CoA in the dimer- and monomer-catalyzed reactions was compared. It has been shown (6) that triacetic acid lactone is generated by the native dimer of 6-hydroxymellein synthase when the enzyme reaction is carried out in the absence of NADPH. The use of malonyl-CoA in the normal and

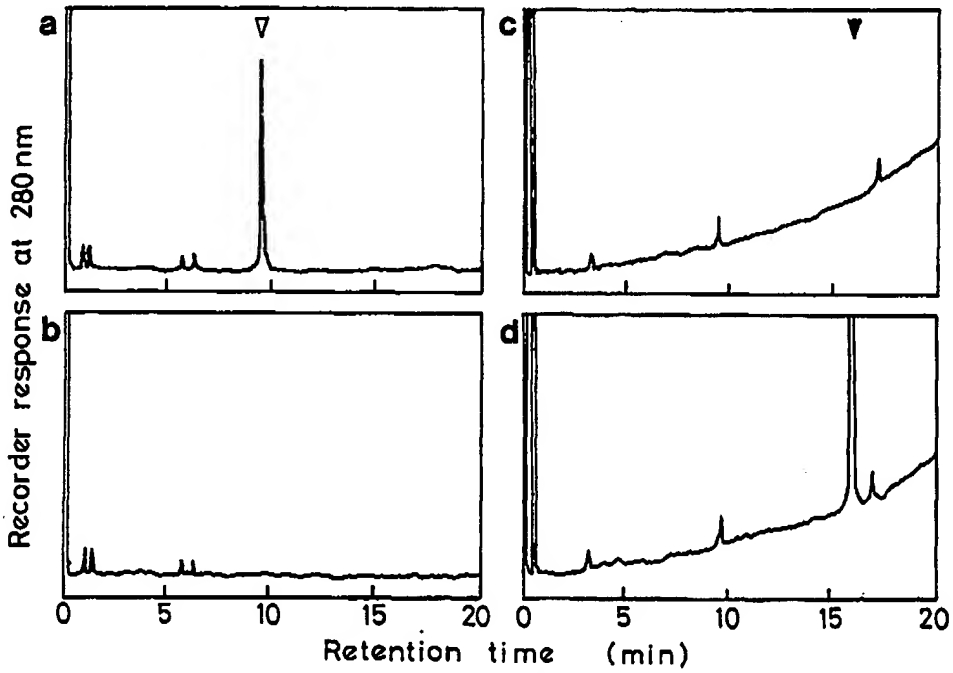

FIG. 2. HPLC analysis of the derailment product liberated by monomeric 6-hydroxymellein synthase. Monomeric (a, c) or dimeric (b, d) forms of 6-hydroxymellein synthase were incubated with acetyl-CoA and malonyl-CoA in the presence of NADPH, and the reaction products were analyzed by HPLC on a reversed-phase C_{18} column. Analytical conditions were described under Materials and Methods. Open and solid arrowheads indicate the positions at which authentic triacetic acid lactone and 6-hydroxymellein elute, respectively.

the minus NADPH reactions was found to be quite different, and the incorporation efficiency into the respective products, 6-hydroxymellein and triacetic acid lactone, was estimated to be 1:0.06 (6). In the native dimer- and inactive monomer-catalyzed reactions run in the presence of NADPH, almost the same ratio was obtained for the use of malonyl-CoA in the syntheses of the two products (1:0.07). All these results appeared to rule out the possibility that triacetic acid lactone might be the product of some enzymatic reaction other than that of 6-hydroxymellein synthesis, and, therefore, I conclude that monomeric 6-hydroxymellein synthase liberates triacetic acid lactone as the sole derailment product, instead of 6-hydroxymellein, even in the presence of NADPH. The present results strongly suggest that the NADPH-dependent keto reduction of the triketide intermediate does not take place in the monomeric form of 6-hydroxymellein synthase and that this defect results in the termination of elongation at the triketide stage to liberate triacetic acid lactone (Fig. 1).

DISCUSSION

It was reported previously (12) that a marked deuterium isotope effect was observed in the rate of 6-hydroxymellein synthesis when the reaction was run in the presence of stereospecifically labeled [4-^{2}H]NADPH and, therefore, concluded that this keto-reducing process is a rate-limiting step in the biosynthesis of 6-hydroxymellein. Since the triketomethylene species is expected as the intermediate preceding this slow-reducing reaction, and the reduction must precede the condensation of the third malonyl-CoA, the enzyme-bound form would be expected to be the most abundant among the catalytically active species of the reaction cycle of 6-hydroxymellein biosynthesis. As shown in Table I, acetyl-CoA and malonyl-CoA bound to dimeric 6-hydroxymellein synthase at a molar ratio of 1:2, indicating that the triketide-bound form is the most abundant intermediate in 6-hydroxymellein synthesis. This observation is consistent with our previous suggestion that the hydride transfer from NADPH to a carbonyl group of the triketide is the slowest step among the several reactions in the biosynthesis of the compound. In monomeric 6-hydroxymellein synthase, the same ratio of the enzyme-bound acyl-CoAs was also observed (1:2). This observation implies that, in the dissociated enzyme, the condensation of acetyl-CoA and malonyl-CoA is also relatively fast compared to the release of the triketide as the cyclized product.

Results obtained in the present study indicate that the keto reduction of the triketomethylene chain takes place only in the dimeric form of the synthase. It is reasonable to suggest, therefore, that the catalytic sites of the two partial reactions of 6-hydroxymellein synthase, chain elongation and keto reduction, are able to associate with each other in functional form only in the homodimeric structure. These findings led me to hypothesize that the catalytic domain of ketomethylene chain elongation would associate with that of keto reduction belonging to another subunit in the homodimer structure (Fig. 3). This assumption also implies that two reaction centers are organized in each molecule of the active enzyme and might agree with the observation that the dimeric molecule of the synthase is capable of binding twice the number of the substrates compared with the monomer subunit (Table I). This hypothetical model of the synthase active site resembles type IA fatty acid synthases of animal cells (3, 4) in which two multifunctional subunits are aligned in an

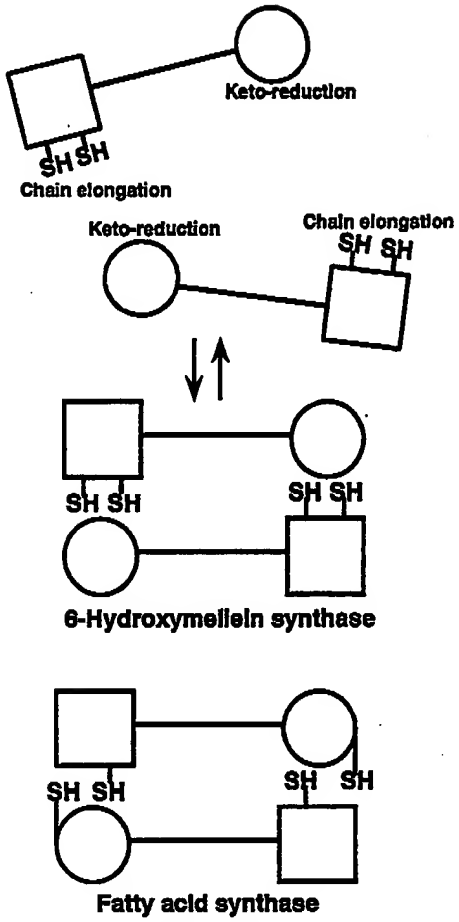

FIG. 3. Schematic presentation of the hypothetical arrangement of the functional domains of 6-hydroxymellein synthase. The catalytic domain for ketomethylene chain elongation is able to associate with that of keto reduction belonging to another subunit in the homodimer, and two reaction centers are organized in each molecule of the active synthase. Although the organization of the homodimer of 6-hydroxymellein synthase and type IA fatty acid synthase is similar, the detail arrangement of the functional domains in the peptide chain, especially the location of the SH group contributed from acyl carrier protein, is likely to be different.

antiparallel direction to form two complete reaction centers. However, unlike 6-hydroxymellein synthase, it was demonstrated (14) that the monomer of type IA fatty acid synthase lacks the acyl-CoA condensation reaction, but not keto reduction, when the partial activities were assayed in the presence of the appropriate substrates. At present, it is widely accepted (3, 14–18) that one of the two SH groups at the reaction center of type IA fatty acid synthase belongs to the other subunit of the homodimer. This SH group is derived from 4′-phosphopantetheine attached to acyl carrier protein and is located near the keto reduction domain (Fig. 3). In contrast, in 6-hydroxymellein synthase, the SH group from acyl carrier protein (8) would be expected to be involved in the reaction center of the acyl-CoA condensation, even in the dissociated form (Fig. 3). These facts clearly indicate that the contribution and arrangement of the catalytic domains in the reaction center of 6-hydroxymellein synthase are different from those of type IA fatty acid synthases, even if the overall organization of these two related enzymes is similar. Further elucidation of the homodimeric structure of 6-hydroxymellein synthase is in progress in my laboratory.

REFERENCES

1. Leistner, E. (1981) *in* The Biochemistry of Plants: Biosynthesis of Plant Quinones (Stumpf, P. K., and Conn, E., Eds.), Vol. 7, pp. 403–423, Academic Press, New York.
2. Luckner, M. (1972) Secondary Metabolism in Plants and Animals, Academic Press, New York.
3. Wakil, S. J. (1989) *Biochemistry* **28**, 4523–4530.
4. Wakil, S. J., and Stoops, J. K. (1983) *in* The Enzymes: Structure and Mechanism of Fatty Acid Synthetase (Boyer, P., Ed.), Vol. 16, pp. 3–61, Academic Press, New York.
5. Kurosaki, F., and Nishi, A. (1983) *Phytochemistry* **22**, 669–672.
6. Kurosaki, F., Kizawa, Y., and Nishi, A. (1989) *Eur. J. Biochem.* **185**, 85–89.
7. Kurosaki, F., and Nishi, A. (1988) *FEBS Lett.* **227**, 183–186.
8. Kurosaki, F., Itoh, M., Yamada, M., and Nishi, A. (1991) *FEBS Lett.* **288**, 219–221.
9. Kurosaki, F. (1995) *Phytochemistry*, in press.
10. Kurosaki, F., Matsui, K., and Nishi, A. (1984) *Physiol. Plant Pathol.* **25**, 313–322.
11. Collie, J. N. (1891) *J. Chem. Soc.* **59**, 607–617.
12. Kurosaki, F., Itoh, M., Kizawa, Y., and Nishi, A. (1993) *Arch. Biochem. Biophys.* **300**, 157–163.
13. Laemmli, U. K. (1970) *Nature* **227**, 680–685.
14. Stoops, J. K., Ross, P., Arslanian, M. J., Aune, K. C., Wakil, S. J., and Oliver, R. M. (1979) *J. Biol. Chem.* **254**, 7418–7426.
15. Stoops, J. K., and Wakil, S. J. (1981) *J. Biol. Chem.* **256**, 5128–5133.
16. Tsukamoto, Y., and Wakil, S. J. (1988) *J. Biol. Chem.* **263**, 16225–16229.
17. Morris, S. M., Nilson, J. H., Jenik, R. A., Winberry, L. K., McDevitt, M. A., and Goodridge, A. G. (1982) *J. Biol. Chem.* **257**, 3225–3229.
18. Stoops, J. K., Wakil, S. J., Uberbacher, E. C., and Bunick, E. J. (1987) *J. Biol. Chem.* **262**, 10246–10251.